The Influence of Iron on the Cellular Quota of Prochlorococcus

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LONG-TERM GOALS

This project studied the influence of iron nutrition on the physiology and molecular ecology of marine cyanobacteria and their resultant effects on seawater optical properties. The influence of iron on marine primary production is now known to rival that of nitrogen. Given iron's integral part of photosynthetic metalloproteins, this element can have a strong effect on the pigmentation of phytoplankton. The marine cyanobacteria, including *Prochlorococococcus marinus* and marine *Synechococcus*, are among the most abundant phytoplankton in the oceans, and hence contribute significantly to both carbon cycling and ocean optical properties. Through this Office of Naval Research Young Investigator Award (ONR-YIP), our laboratory has studied the iron requirements of these marine cyanobacteria and their physiological, optical and molecular characteristics. Because *Prochlorococcus* is relatively difficult to grow and pure cultures were only recently produced (Saito et al., 2002), studies of the elemental composition and requirements of this key phytoplankton species have not been possible until recently. Yet, global ecosystem models have been lacking necessary iron growth parameters for this key phytoplankton group that are needed to improve the predictive capability of marine primary productivity and the seawater optical properties (Aumont et al., 2003; Moore et al., 2002; Moore et al., 2004; Schmittner et al., 2005; Weber et al., 2007).

OBJECTIVES

This project was focused on studying the influence of iron on marine cyanobacteria and the resultant implications for optical properties and primary productivity. Specific objectives included: measuring the iron requirement of *Prochlorococcus* through physiological experimentation, quantifying the iron and carbon content of *Prochlorococcus* under a range of iron and light conditions, quantifying the dissolved organic matter production from iron limited cultures, examining the pigment per cell using flow cytometry, and examining the genomic and proteomic responses to iron stress in *Prochlorococcus* and *Synechococcus*, respectively. In addition field work was conducted to examine the distribution of *Prochlorococcus* in comparison with dissolved iron concentrations, as well as to survey for iron limitation of phytoplankton in marine environments not previously recognized to be iron limited.

APPROACH

We have undertaken careful physiological studies of the iron growth requirements in representative *Prochlorococcus* strains, and have made concurrent measurements of their cellular iron quotas using inductively coupled plasma mass spectrometry (ICP-MS). Growth studies were being performed using

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cultures of *Prochlorococcus* and media prepared in a trace metal cleanroom, which prevents dust particles from contaminating cultures for the metal limitation conditions needed. To complement these studies, analyses of the whole genomic response of *Prochlorococcus* under iron stress were performed using microarray technology. This work has largely been being carried out by graduate student Anne Thompson, a MIT-WHOI Joint Program student in Biology, co-advised by Saito and Sallie Chisholm (MIT). Alysia Cox is a MIT-WHOI Joint Program student in Marine Chemistry and Geochemistry who is also working on ancillary studies of cadmium utilization by marine cyanobacteria.

WORK COMPLETED

Physiological experiments were completed on *Prochlorococcus* strain MED4 and MIT9313 under a range of iron treatments. Samples were analyzed for growth rate, carbon and iron content, DOM production, and fluorescence per cell. In addition genomic microarray analyses were conducted on two strains of *Prochlorococcus* MED4 and MIT9313 (Thompson et al in preparation). Field samples from an R/V Knorr cruise in 2005 (R/V Knorr, Saito Chief Scientist) were analyzed for *Prochlorococcus* and iron concentrations (Thompson et al in preparation). Improvements on the analysis of dissolved iron using inductively coupled mass spectrometry analysis (ICP-MS) were made and published (Saito and Schnieder, 2006). Cadmium uptake methodologies were also developed using stable isotope additions and ICP-MS (Cox and Saito, in prep.). A synthesis and review manuscript on the colimitation of marine phytoplankton by multiple nutrients was written (Saito et al., 2008), that is intended to help frame and provide definitions and context for the complex subject of metal colimitation in marine phytoplankton.

A second research cruise (R/V Knorr, Saito Chief Scientist) was conducted in 2007 across the South Atlantic Gyre into the Benguela Upwelling system. This coastal system was demonstrated to be iron limited for the first time, consistent with previous observations that the Californian, Peruvian, and Arabian upwelling systems can experience iron limitation of phytoplankton. In addition the South Atlantic Gyre was shown to be iron-light, iron-nitrogen, or cobalt-nitrogen limited in three distinct experiments and locations, indicating the importance of iron (and cobalt) even in regions previously recognized to be controlled by nitrogen limitation (e.g. modeling predictions in Moore et al., 2004). Together these results demonstrate that iron can be an important controller of phytoplankton productivity through decreases in phytoplankton pigment caused by iron stress (see above).

Finally, genomic and proteomic analyses were conducted on marine cyanobacteria focusing on iron nutrition. Whole genome microarrays techniques were applied to iron limitation experiments of *Prochlorococcus*. "Shotgun proteomic" analyses of marine *Synechococcus* were also completed using nanospray liquid chromatography mass spectrometry in the Saito laboratory (Saito et al., under revision). This proteomic work is also supported by a 2008 ONR-DURIP equipment award to Van Mooy and Saito.

RESULTS

During this Office of Naval Research Young Investigator Award, we have conducted a study of the influence of iron on marine cyanobacteria through laboratory experimentation and field analyses. Numerous findings and at least 6 papers will have resulted from this grant (two already published-see above, 1 in revision, 3 in preparation). These results are organized into laboratory and field projects and are summarized below.

Laboratory experiments on the iron physiology of requirements of *Prochlorococcus* and *Synechococcus* can be grouped into six major findings. First, physiological experiments show that iron requirements of *Prochlorococcus* appear to be lower than the estimated quantities of available free iron in seawater (Figure 1). In seawater iron is complexed by strong organic ligands, and the iron bound by those ligands is believed to be harder to acquire for phytoplankton than the free (unbound, or more precisely, weakly bound) forms of iron (Rue and Bruland, 1997). These experiments imply that *Prochlorococcus* must be able to utilize the ligand-bound iron in order to maintain their growth rates. This is an important finding that has broad implications for our understanding of *Prochlorococcus* growth and iron cycling in the oceans. Second, our preliminary cellular iron quota measurements suggest that there is an effect of light on the content of iron within the cell, consistent with the high iron requirement of the Photosystem I apparatus. Third, analysis of pigment per cell under iron limitation shows that *Prochlorococcus*, despite having a "minimal genome" and being the smallest photosynthetic cell known, displays a significant decrease in fluorescence per cell (>30%) under iron stressed conditions by greatly decreasing its pigmentation (Figures 2 and 3). This phenomenon, known as chlorosis, is found in many land plants and larger phytoplankton cells but this is the first laboratory documentation of it occurring in *Prochlorococcus*. ***Given the widespread distribution of both Prochlorococcus and iron limitation in the oceans (see field results below), Prochlorococcus' display of iron induced chlorosis has important implications for the global optical properties of seawater.***

Fourth, whole genome microarray studies demonstrate a number of genes that are affected by iron limitation in the *Prochlorococcus* strain MED4 (examples of iron responsive genes shown in Figure 4). Knowing the identity of genes that are influenced by iron is important for several reasons: first, they assist in understanding how the physiology of *Prochlorococcus* is affected by iron nutrition; second, they provide crucial information about candidate genes that can be utilized as biomarkers of iron stress. Many of these genes that respond to iron limitation do not currently have a known function, when compared to current genomic and biochemical knowledge. As a result, they may be genes that are involved in the utilization of the ligand-bound iron described above. Figure 4 shows an example of two genes that respond strongly to iron limitation.

Fifth, "shotgun proteomics" analyses of marine *Synechococcus* WH8102 were completed identifying 626 proteins, comprising 25% of the genomically coded open reading frames (Saito et al., in revision). This analysis was conducted in the Saito laboratory using nanospray liquid chromatography mass spectrometry and is also supported by a recent ONR-DURIP award for a triple quadrapole mass spectrometer. There are many findings from this study, and it is a major launching point for further scientific inquiry. From a Navally-relevant perspective, the most important finding is that the Photosystem I:Photosystem II ratio in *Synechococcus* as determined by proteomic quantification of all identified proteins from each photosystem was close to 1:1, rather than the historically predicted 3:1 (Kustka et al., 2003; Raven, 1990). This finding has major implications for the predicted iron use efficiency and potential for chlorosis pigmentation effects in marine Cyanobacteria because Photosystem I requires a large number of iron atoms. Our conception of the iron requirements of marine Cyanobacteria and their marine ecology will have to be rethought based on this finding.

Sixth, as described above the interactions between iron and light are inseparable with phytoplankton because of the crucial role of iron metalloproteins in the photosynthetic apparatus. From a field perspective iron and light colimitation is believed to be one of the most important "colimitations" that

exist in the oceans (Maldonado et al., 1999). The term colimitation usually refers to instances where two nutrients are limiting simultaneously. However, significant confusion surrounds the variety of types of colimitation. We have written a synthesis/review manuscript that defines three types of colimitation based on biochemical relationships: independent, biochemical substitution, and biochemical dependence (Saito et al., 2008). These colimitations were described in terms of modified Monod-type equations and represented visually as three dimensional images (Figure 5). Iron-light colimitation is a complex scenario since iron has long been known to be important in light acquisition, in particular in the iron-rich Photosystem I complex. Because of this functional use of iron in light acquisition we have defined "iron on light colimitation" as a Type III Biochemically dependent system. Interestingly, this is significantly different than the current parameterizations of Type I Biochemically independent used in global ecosystem models (Aumont et al., 2003; Moore et al., 2004), and suggests the need for the modeling community to correct their colimitation algorithms to accurately represent the biochemistry of iron-light colimitation.

Recent field experiments from 2007 R/V Knorr cruise have also been conducted as part of this research grant. These field based findings are highly synergistic with the laboratory findings described above. There are three key findings based on our field efforts that pertain to this study. First, in Figure 6 we present the first data demonstrating that the Subtropical Gyre of the South Atlantic can be limited by iron at the base of the euphotic zone (Saito et al., in prep). This area is consistently predicted to be nitrogen limited in ecosystem models (Aumont et al., 2003; Moore et al., 2002; Moore et al., 2004), yet these bottle incubation results demonstrate the importance of iron limitation at the base of the euphotic zone in this region. Second, in Figure 7 we observe two types of colimitation at another location within the Subtropical Gyre of the South Atlantic. Cobalt-nitrogen colimitation was observed at 10m depth, while iron-nitrogen colimitation was observed at 40m at the same location. The vertical distributions of trace metals iron and cobalt are significantly different than that of the macronutrients. Because iron and cobalt are subject to chemical and biological scavenging, these elements tend to become depleted in intermediate and deep waters (Noble et al., 2008). As a result of the differing vertical profile structure of nitrate, iron and cobalt, the relative stoichiometries of these required nutrients varies with depth, priming the ecosystem for distinct and complex limitations and colimitations with depth as we observed (Figure 7). Third, we conducted bottle incubations in the Benguela Upwelling system off of Africa and found this region to be iron limited in regions of low dust deposition. This is the first finding of iron limitation in this upwelling system, and is consistent with findings of iron limitation in the Californian (Hutchins et al., 1998), Peruvian (Hutchins et al., 2002), and Arabian upwelling systems (J. Moffett pers.comm.), suggesting that iron limitation of these coastal upwelling waters is a global phenomenon. **Given the strong influence of iron on phytoplankton pigmentation described above (Figures 2 and 3) and in the literature, these findings of iron limitation and colimitation in coastal and open ocean oligotrophic gyres suggest iron limitation should affect the pigmentation properties of phytoplankton globally in many different marine environments.**

Three manuscripts have already been published from this grant (Noble et al., 2008; Saito et al., 2008; Saito and Schneider, 2006), one is in review (Saito et al., *Synechococcus* proteomics), and four more are in preparation (two Thompson et al.: iron-*Prochlorococcus* physiology and iron-*Prochlorococcus* microarray analyses; and two field based manuscripts based on the recent 2007 South Atlantic cruise). In summary, this study is the first comprehensive examination of iron and the marine cyanobacterium *Prochlorococcus* and has provided important information about the iron requirements, changes in cellular fluorescence under iron limitation, gene regulatory response related to iron stress, proteomic

analyses and implications for iron cellular quotas, the parameterization of iron-light colimitation in ecosystem models, and observations of iron limitation and other colimitations in coastal and open ocean environments.

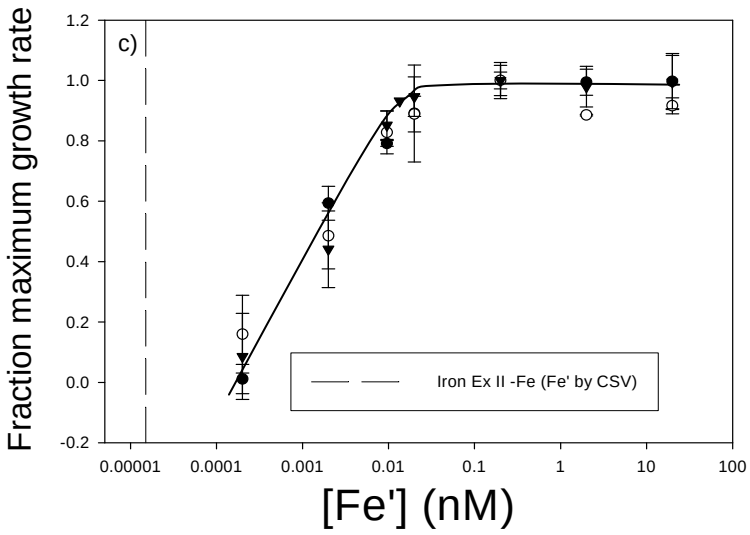

Figure 1. Laboratory experiments demonstrating the limitation of Prochlorococcus growth by iron. Fe' is representative of the "free" iron in seawater, and refers to the concentration of all inorganic species of iron (including free ions, Fe^{3+}, and hydroxo and chloro complexes). The vertical dashed line indicates the concentration of Fe' measured in the oceans by electrochemical techniques (Rue and Bruland, 1997). Comparison of these two results clearly demonstrates that Prochlorococcus cannot maintain reasonable growth rates if only Fe' is available. Hence this data set is strong evidence for the utilization of the organically complexed iron in seawater, which exists in much higher concentrations. The genetic/chemical mechanism for removal of iron from these complexes by cyanobacteria is currently unknown, but genomic experiments should shed useful insight into these mechanisms (e.g. Figure 4). From Thompson et al., in prep.

[Graph of fraction of maximum growth rate versus Fe', where fraction of maximum growth rate is ~1 between 30nM down to 0.1nM and begins to decrease at ~0.01nM and resulting in little to no growth below 0.001nM. The dashed line representing Fe' in the Pacific is much lower than the lowest Fe' needed to support Prochlorococcus growth, indicative of the use of organically complexed iron.]

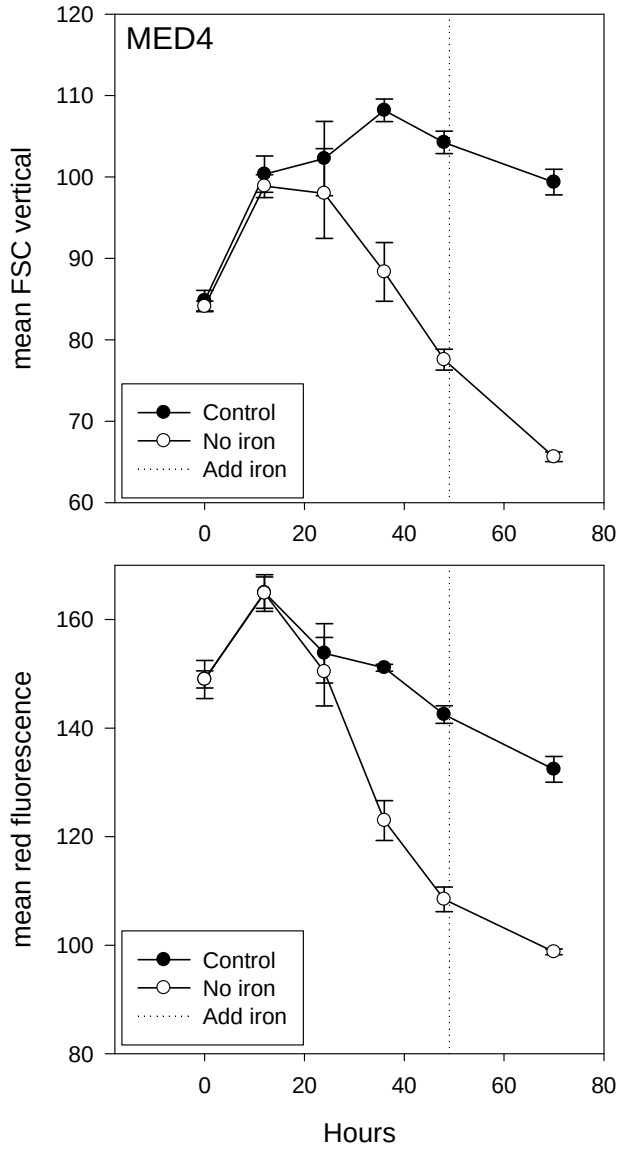

Figure 2. Evidence for a large decrease in cell size (top panel) and photosynthetic pigments (bottom panel, ~30% decrease in fluorescence) in Prochlorococcus caused by iron limitation. Iron specifically plays a major part in influencing phytoplankton pigmentation properties because of its important role within the (iron) metalloproteins of Photosystem I. Prochlorococcus is known to be limited by iron in the equatorial Pacific, but is likely also iron limited at the base of the euphotic zone in other regions previously thought to only be limited by nitrogen (e.g. the South Atlantic see Figure 6).

[Top graph displaying forward light scatter as an indication of cell size versus time where no added iron treatment decreases from ~100 to 65 after 70 hours. Bottom graph displaying red fluorescence versus time where no iron treatment drops from ~165 to 100 after 70 hours]

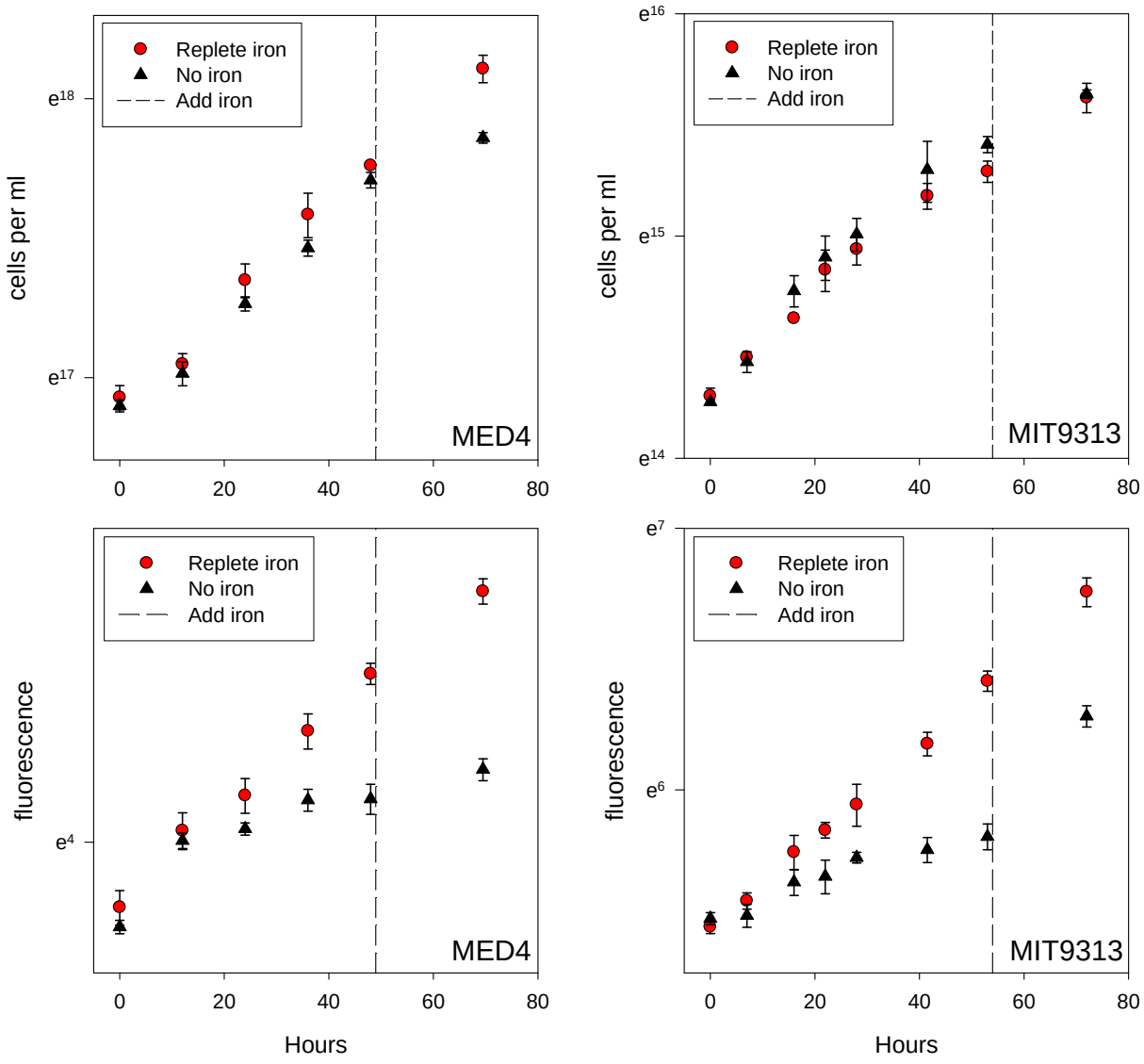

Figure 3. Additional experimentation demonstrating a significant decrease in photosynthetic pigments in Prochlorococcus caused by iron limitation. Top panels show cell numbers in experiments continuing to increase, while bottom panels show major losses in pigments (as measured by fluorescence) caused by iron limitation for two strains of Prochlorococcus. Both experiments show a recovery of pigments after addition of iron (dashed line).

[Top graphs displaying increasing cell numbers in iron replete and iron limited cultures. Bottom panels showing increases in fluorescence in iron replete cultures, but a plateau of fluorescence after 30 hours due to iron limitation.]

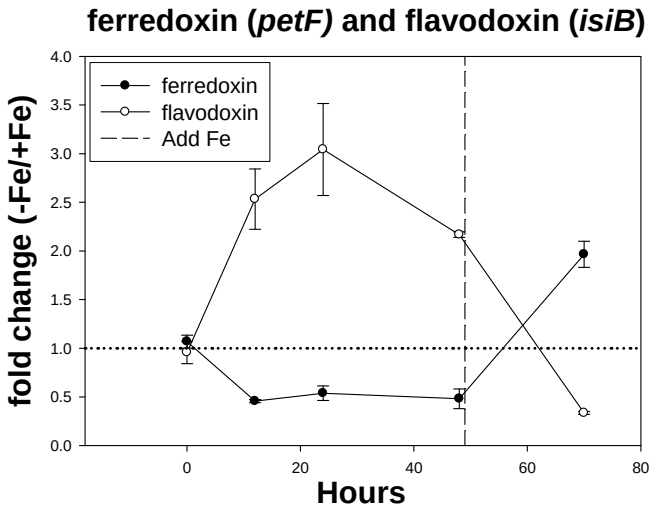

Figure 4. Whole genome microarray data for selected genes in Prochlorococcus MED4 demonstrating increased flavodoxin transcripts (which does not contain iron) and decreased ferrodoxin transcripts (which contains iron) under iron stress. Upon iron addition (vertical dashed lines), transcripts signaling the production of each compound reverse, demonstrating a clear iron effect on these genes. Many other genes were also found to respond to iron stress and additions.

[Graph displaying "fold change of the iron deplete treatment relative to iron replete treatment" relative to time (70 hour experiment), where ferredoxin is at ~0.5 and flavodoxin goes as high as 3.0. When iron is added at 50h to the –Fe treatment the trend reverses where ferredoxin increases to ~2 and flavodoxin decreases to ~0.5.]

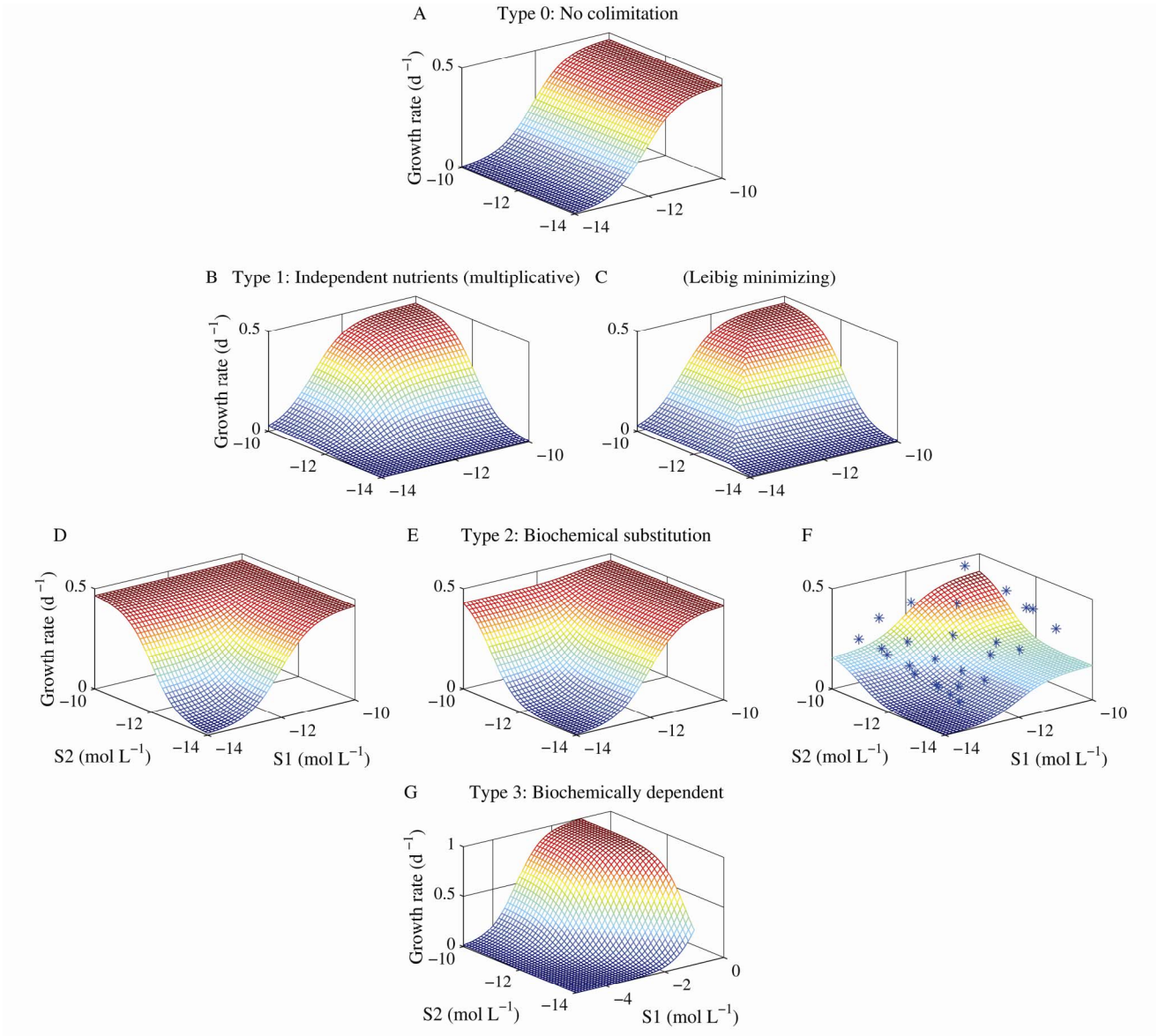

Figure 5. Three-dimensional representations of colimitation scenarios for marine phytoplankton, where S_1 and S_2 are the substrate nutrients. The concept of colimitation has not been rigorously defined until this study, and iron-light colimitation is likely the most important colimitation in the marine environment (Maldonado et al, 1999). (A) Type 0 no colimitation concerns two elements, where only one is a nutrient. Type I independent nutrients concerns two nutrients that do not share a biochemical function, such as nitrogen and phosphorus. Two expressions of Type I are plotted: (B) the multiplicative form, and (C) the minimization (Leibig) form. Type II biochemical substitution concerns two micronutrients that can substitute for the same biochemical function, usually due to a metalloenzyme that can be active with two different metals (e.g., Zn and Co). Three scenarios are presented, (D) where two nutrients substitute perfectly for each other, (E) where the two nutrients have unique half saturation constants, and (F) where two nutrients only partially substitute for each other, leaving a non-substitutable component of each biochemical quota. In this case, maximal growth occurs when both nutrients are present, representing a situation where the cambialistic metalloenzyme constitutes only a fraction of the total S_1 and S_2 quotas. (G) Type III

biochemically dependent colimitation concerns two nutrients where the acquisition of one (S_1) is dependent on the sufficient nutrition of the other (S_2) (e.g., C and Zn or Fe and light). Iron and light limitation are believed to be biochemically dependent (Type III), due to the significant number of iron atoms found within the enzymes of photosystem I. Our data from Prochlorococcus microarray studies demonstrate the response of photosystem I to iron limitation, consistent with this assignment of Type III for iron and light. Importantly, ecosystem models instead use the Type I multiplicative colimitation parameterization (B) for iron-light colimitation, and likely should be changed to reflect these findings. From Saito et al., Limnol. Oceanogr. 2008.

[Three dimensional graphs with a surface that represent examples of each of the four types of colimitation in marine phytoplankton (Type 0, I, II, and III). Each type has a unique surface that relates to the biochemical nature of the colimitation. Growth rate is in the vertical axis, and substrate 1 and 2 (S1 and S2) are in the x and y axes. In Type 0 growth rate is high when S1 is high and does not respond to variations in S2. In Type I growth rate is highest when both S1 and S2 are high. Growth rate is low only when either S1 or S2 are low. In Type II growth is high when both S1 and S2 are high, and when either S1 and S2 is high. Growth rate is low only when both S1 and S2 are low. In Type III growth rate is high when both S1 and S2 are high, and low when either S1 or S2 is low. But in this case, more S1 is needed when S2 is low due to the biochemical dependence effect of S1 acquisition on S2.]

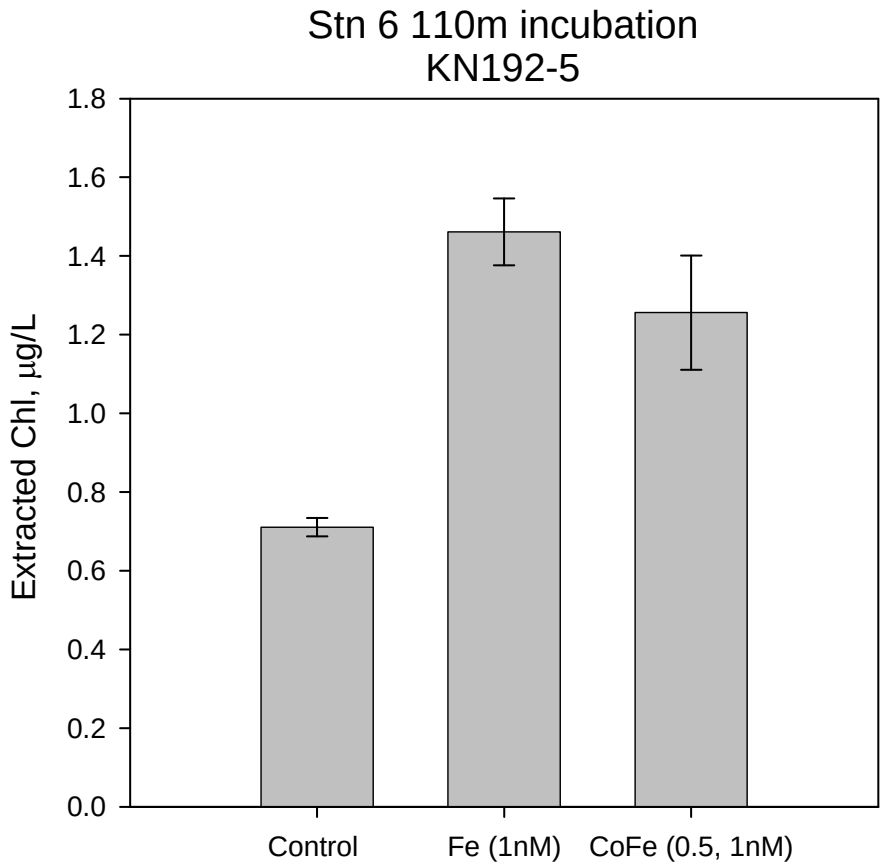

Figure 6. Iron limitation of the phytoplankton community at 110m depth within the South Atlantic gyre, November 2007. This region is consistently predicted in ecosystem models to be nitrogen limited (e.g. Moore et al., 2004), yet these results clearly show at this depth iron limitation is an important factor controlling primary productivity.

[Graph showing extracted micrograms of chlorophyll per liter relative control, plus 1nM iron, and plus 0.5nM cobalt and 1nM iron in triplicate. Both treatments containing iron show significantly more chlorophyll at the end of the experiment than the control, with averages of 0.71, 1.46 and 1.26 micrograms chlorophyll per liter, respectively.]

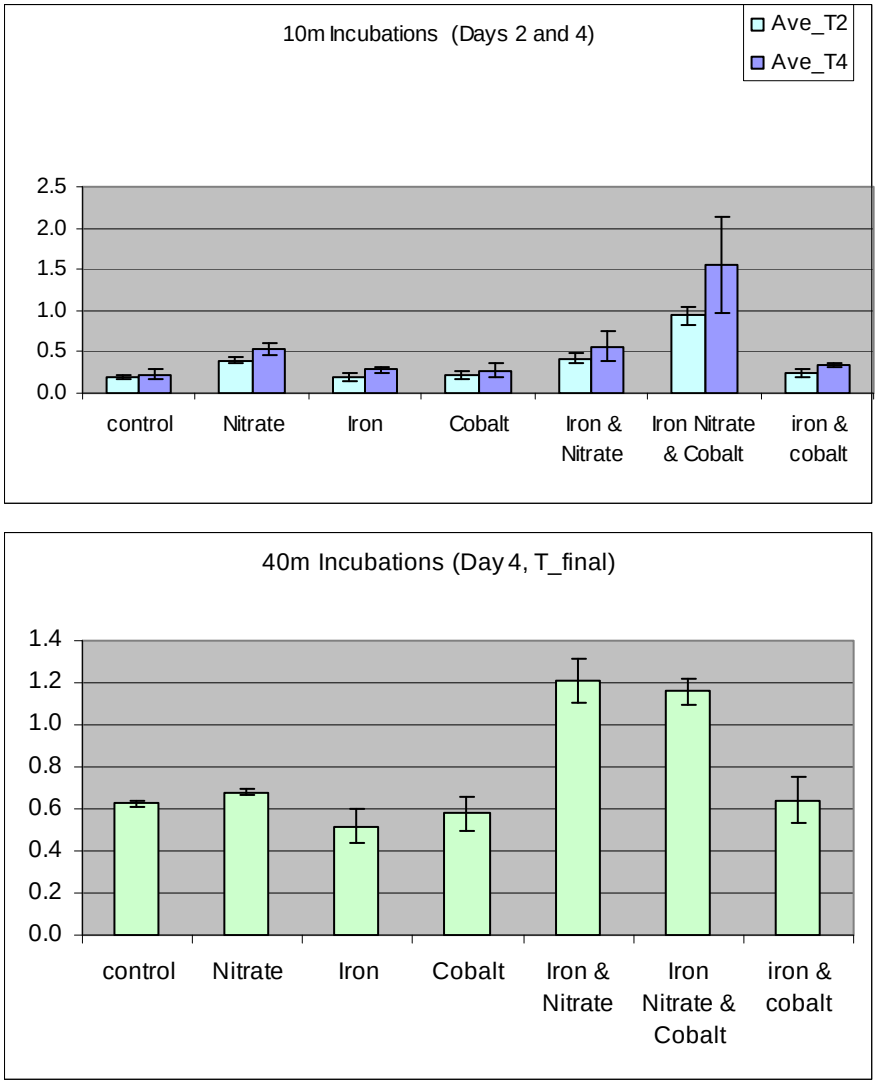

Figure 7. Colimitation of primary productivity in the South Atlantic Gyre, December 2007. Top panel shows a bottle incubation from 10m depth where increases in extracted micrograms chlorophyll per liter for nitrate and iron+nitrate, but a much greater stimulation for iron+nitrate+cobalt. Because iron+nitrate is similar to the nitrate treatment, this experiment demonstrates nitrate-cobalt colimitation at this depth. Bottom Panel shows iron-nitrate colimitation but little response to iron, nitrate, or cobalt independently. Together these results demonstrate the importance of micronutrients in controlling productivity in regions currently predicted to be only nitrogen limited. Moreover they demonstrate the importance of the phenomenon of colimitation (See Figure 5).

[Top graph showing extracted micrograms of chlorophyll per liter in triplicate for seven treatments from water collected at 10m depth: control, nitrate, iron, cobalt, iron and nitrate, iron nitrate and cobalt, and iron and cobalt. Nitrate and iron-nitrate treatments show a moderate stimulation relative to the control, while the iron-nitrate-cobalt treatment shows a large stimulation. Bottom panel, same experiment and treatments but water was collected from 40m depth. Iron-nitrate and iron-nitrate-cobalt show significant stimulation relative to the control. None of the other treatments show a significant response relative to the control.]

IMPACT/APPLICATIONS

We anticipate that our iron growth requirement data for *Prochlorococcus* will be important parameters in coupled Ecosystem-Global Circulation Models (e.g. Aumont et al., 2003; Moore et al., 2002; Moore et al., 2004; Schmittner et al., 2005; Weber et al., 2007). In addition, the significant decrease in pigment in *Prochlorococcus* under iron limitation (30% as measured by fluorescence per cell flow cytometric analyses) demonstrates iron limition strongly affects the optical properties of *Prochlorococcus*. A synthesis-review manuscript of colimitation should be useful in the correctly framing the phenomenon of iron-light colimitation in ecosystems models. Finally, field observations of iron limitation and various colimitations demonstrate important phenomenon currently overlooked by ecosystem models.

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HONORS/AWARDS/PRIZES

2005 Ruth and Paul Fye Best Paper Award in Chemical Oceanography – WHOI
2007 Invited Speaker – US Ocean Carbon and Biology Workshop
2008 Invited Speaker - Gordon Conference on Environmental Bioinorganic Chemistry
2008 Keynote Speaker – Medical University of South Carolina, Annual Student Open House